Final Report

Project No. 00-HHE-2a

Overcoming molecular sample processing limitations: New platform technologies

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Abstract

Rapid, low-cost extraction of DNA from bacterial cultures and colonies was investigated using four reagents: Instagene matrix (Bio-Rad, Hercules, CA); Isocode paper (Schleicher and Schuell, Keene, NH); PrepMan (Applied Biosystems, Foster City, CA); and Xtra Amp tubes (Ansys Diagnostics, Lake Forest, CA). Prices per sample ranged from under 50¢ for Isocode paper to $1.80 for Xtra Amp. Using *E. coli* O157:H7 as the test organism, all methods generated PCR-quality DNA; the fastest and most user-friendly was the Xtra Amp reagent. The most versatile was the Instagene matrix, which successfully extracted DNA from other, gram-positive bacteria such as *Listeria monocytogenes*. The Instagene matrix also extracted DNA from lysates of *Cryptosporidium parvum* and *Giardia lamblia*.

Continuous flow centrifugation (CFC) was evaluated for the recovery of *C. parvum* oocysts spiked into 10 L volumes of source water. The procedure took about 2.5 hours to conduct, followed by an overnight immunomagnetic separation (IMS) step using Dynal beads (Dynal, Lake Success, NY). For spiking experiments using 100 oocysts, 9 of 10 replicates were positive using immunofluorescence microscopy (IFA) with the MeriFluor reagent (Meridian Diagnostics, Cincinnati, OH), (mean recovery of 4.4 oocysts, range 3 to 8). Another 10 replicates relied on nested PCR for the *C. parvum* TRAP C-1 and Cp41 genes; again, 9 of 10 replicates were positive. When the spiking dose was reduced to 10 oocysts in 10 L of raw water, 10 of 12 replicates (83%) were positive, with a mean recovery of 3.2 oocysts (range 1 - 12).

Several techniques were evaluated for use in rapid extraction of RNA from a bovine cell line (MDBK cells) infected with bovine enterovirus (BEV). An automated platform for nucleic acid extraction, the ABI 6100 instrument (Applied Biosystems, Foster City, CA), did provide RT-PCR quality RNA from cells, but the yield was substantially lower than with the more labor-intensive Qiagen Viral RNA kit (Valencia, CA). The ABI 6100 was able to successfully extract DNA from bacterial cultures and colonies, as determined by PCR. Another rapid extraction method, the Xtra Amp RNA tubes, was evaluated, but as with the ABI 6100, yields of RNA were inferior compared to the Qiagen method.

KEY WORDS: DNA, RNA, rapid extraction, *E. coli* O157:H7, *Cryptosporidium parvum*, *Giardia lamblia*, continuous flow centrifugation

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List of Benefits

• Four methods for rapid, inexpensive, and user-friendly extraction of DNA from *E. coli* O157:H7 broth cultures and agar colonies were evaluated.

• The Instagene matrix (marketed by Bio-Rad, Hercules, CA) also provided PCR-quality DNA from gram positive bacteria such as *Listeria*, as well as from lysates of encysted protozoa such as *Cryptosporidium parvum* and *Giardia lamblia*.

• Continuous flow centrifugation (CFC) was evaluated for recovery of *C. parvum* oocysts and *Giardia intestinalis* cysts from spiked, 10 L volumes of raw water.

• The ABI 6100, an automated instrument for nucleic acid extraction from a variety of sample types, was evaluated using cultured cells infected with bovine enterovirus and cultures of different genera of pathogenic bacteria.

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Executive Summary

One of the principal obstacles to the widespread adoption of molecular biology-based methods for the detection of waterborne pathogens is the requirement for purified nucleic acids (DNA and RNA) from what are often complex sample matrices: treated water, raw water, filtrate from water samples, as well as encysted protozoa such as *Cryptosporidium parvum* and *Giardia lamblia*. This project investigated several methods (Xtra Amp tubes, Isocode paper, Instagene matrix, and PrepMan reagent) to extract nucleic acids from a variety of organisms, with the goal of identifying methods that allowed 10 samples to be processed in 45 minutes or less, were reasonably priced on a per-sample basis, and could be performed by individuals without extensive training in molecular biology techniques. With *E. coli* O157:H7 from overnight broth cultures or colonies lifted from agar plates, all of the four methods provided DNA in satisfactory quantity and quality for immediate use in PCR assays. The price per assay ranged from under 50¢ for the Isocode paper to $1.80 for the Xtra Amp tube.

The Instagene matrix was the most versatile of the four methods evaluated, and was used to extract DNA from other genera and species of bacteria, including gram positive *Listeria monocytogenes*, as well as *Salmonella typhimurium* and *Bacillus anthracis* Sterne. When used in conjunction with novel, freeze-dried PCR reagents that simply require reconstitution in sterile water to be used, the time from DNA extraction to real time PCR data was 2.5 hours.

The project also evaluated the use of continuous flow centrifugation (CFC) for the recovery of *C. parvum* oocysts from spiked, 10 L volumes of source water. The CFC technique took approximately 2.5 hours to perform and was coupled with overnight IMS. Recovered oocysts were detected by IFA microscopy, and nested PCR for the Cp41 and TRAP C-1 genes of *C. parvum*. DNA extraction of the recovered oocyst / immunomagnetic bead pellet was accomplished using freeze thawing and Instagene matrix. When a spiking dose of 100 oocysts was used, 9 of 10 replicates were positive by IFA; when another 10 replicates were assayed by nested PCR, again, 9 of 10 were positive. When the spiking dose was reduced to 10 oocysts, 10 of 12 replicates (83%) were positive by IFA; the mean number of recovered oocysts was 3.2. The turbidities of the source water pellet recovered using the CFC technique were extremely high (> 400 NTU).

Because it was found that only nested PCR was capable of detecting small quantities of oocysts, experiments were conducted on purified oocysts measured by a micromanipulator to determine which, if any, primer set could detect 1, 5, or 10 oocysts. Three primer pairs: TRAP C-1, Cp11, and hsp70, were used for PCR and were negative for the three quantities of oocysts. When RT-PCR was used, the hsp70 primers successfully detected the 5 and 10 oocysts, and were faintly positive for the 1-oocyst sample. However, the hsp70 RT-PCR also produced positive results on RNA and DNA extracted from 2 ml aliquots of source water, indicating cross-reactivity with nontarget organisms.

An automated instrument for extraction of nucleic acids from a variety of sample types, the ABI 6100, was investigated using cultured bovine cells infected with BEV. While the RNA produced by the ABI 6100 was suitable for RT-PCR of GAPDH housekeeping gene mRNA, it could not provide sufficient BEV RNA for detection; also, the RNA yield was substantially lower than that achieved with the more labor-intensive Qiagen Viral RNA kit. This was also true of another rapid RNA extraction reagent, the Xtra Amp RNA tube. The ABI 6100 did provide PCR-quality DNA from bacterial overnight broth cultures and colony lifts from an agar plate, but we conclude that its expense limits its utility to those laboratories conducting assays on large numbers of samples (100 or more) a day.

LIST of ACRONYMS

BEV bovine enterovirus

CFC continuous flow centrifugation

DNA deoxyribonucleic acid

GAPDH glyceraldehyde phosphate dehydrogenase

hsp70 heat shock protein (molecular weight) 70 kDa

IFA immunofluorescent antibody

IMS immunomagnetic separation

MBG molecular biology grade

MDBK Madin Darby bovine kidney cells

NTU nephelometric turbidity unit

PBS phosphate buffered saline

PCR polymerase chain reaction

PFU plaque forming units

RNA ribonucleic acid

rRNA ribosomal ribonucleic acid

RT-PCR reverse polymerase chain reaction

TPI triose phosphate isomerase

TRAP C-1 thrombospondin-related adhesive protein (type) C-1

INTRODUCTION

This Final Report summarizes work performed under the Water Environment Research Foundation's project No. 00-HHE-2a, "Overcoming Sample Processing Limitations: New Platform Technologies," by Dr. James Higgins, United States Department of Agriculture, Beltsville, Maryland.

There were several components to the project: one involved evaluation of several low cost, user-friendly methods for rapid extraction of PCR-quality DNA (and RNA, in the case of viruses and bovine cells) from bacterial colonies on agar plates, bacterial broth cultures, oocysts of *Cryptosporidium parvum*, cysts of *Giardia lamblia*, bovine enterovirus and bovine cells in in vitro culture. Another component investigated the use of continuous flow centrifugation (CFC) as a method for recovery of oocysts and cysts from spiked 10L volumes of source (raw) water. Finally, an automated instrument, the ABI 6100 Nucleic Acid Prepstation, was evaluated for its ability to extract RNA from bovine cells in culture, infected with bovine enterovirus.

MATERIALS AND METHODS

1.1 Extraction of DNA from bacteria.

Overnight cultures of bacterial stocks were done in LB broth, trypticase soy broth, or minimal lactose broth, at 37° C on a rocking platform. The tubes containing the cultures were centrifuged at a medium speed (1,000 x *g*) on a tabletop centrifuge to pellet the cells. All but 100 μl of supernatant was discarded and the pellet resuspended. Aliquots were removed and processed as described below.

To determine the sensitivity of enterohemorrhagic *E. coli* - specific PCR, when performed on DNA extracted from a mixed population of *E. coli* using the Instagene matrix, a stock concentration of 10^8 cells / ml of *E. coli* O157:H7 was made using a stationary-phase culture in MLB broth treated with 0.1% sodium azide. Cell numbers were calculated by observation of aliquots of the 10^6 dilution in a hemacytometer using phase contrast microscopy at 400X. This stock concentration was then added to MLB broth, containing *E. coli* ATCC No. 11775 cells at 10^8 cells / ml, to give a dilution series of *E. coli* O157:H7 cells at 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 cells / ml in a constant background of 10^8 *E.*

coli 11775 cells / ml. The dilutions were vortexed to thoroughly mix the cells and then 1 ml aliquots removed for extraction with Instagene matrix (below).

Colonies of *E. coli* O157:H7 on blood agar plates were removed with a sterile plastic loop and vortexed in 100 μl volumes of sterile deionized, molecular biology-grade (MBG) water in a microfuge tube to dislodge the bacteria. The sample was then centrifuged, the supernatant discarded, and the pellet resuspended in 30 μl of MBG water and processed as described below.

DNA was extracted from samples using the Instagene matrix according to the manufacturer's protocol. Briefly, a 30 μl sample was suspended in 200 μl of Instagene™ matrix and vortexed, followed by heating at 56° C for 15 minutes. The samples were vortexed again and heated at 100° C for 8 minutes, then centrifuged to pellet the matrix. Aliquots of 10 μl and 20 μl (the recommended amount) were used as template for PCR.

Extraction with the Isocode™ paper followed the manufacturer's instructions (Schleicher and Schuell, Keene, NH). Briefly, 10 μl aliquots of bacterial cultures were spotted directly onto 8 mm diameter disks of the paper and DNA was eluted in a 100 μl volume of MBG water, with 5 - 20 μl used as template for PCR.

Sample processing with the PrepMan™ reagent (Perkin Elmer/ABI, Foster City, CA) involved adding up to 30 μl of the sample to 200 μl of PrepMan reagent, vortexing, and then heating at 100° C for 10 min. The preparation was centrifuged to pellet the matrix and 1-5 μl supernatant used as template for PCR.

For the Xtra Amp® tubes, (Ansys Diagnostics, Lake Forest, CA) up to 30 μl of sample was added to an Xtra Amp tube containing 75 μl of Lysis Buffer, and the total volume brought up to 150 μl with MBG water. The mixture was incubated for 10 minutes at room temperature, then discarded and the tube washed twice with 175 μl of Wash Buffer. The wash buffer was discarded and laboratory tissue papers were used to dry the interior of the tube. The PCR master mix (50 μl, below), including 5 μl of Xtra Amp Enhance reagent, was pipetted directly into the tube and cycling increased by 3 cycles as per the manufacturer's instructions. For real time thermal cycling on the ABI 7700 instrument, the Xtra Amp caps were replaced by optical caps (PE Biosystems, Foster City, CA).

The *E. coli lac Z* probe is: 5' (FAM) CGC CTT ACT GCC TGT TTT GAC (TAMRA or BHQ, a proprietary "black hole quencher" dye marketed by Biosearch International, Novato, CA), which spans nucleotides 5963-5986 of Genbank Accession No. AE000141.1; and the primers are: *lac Z* forward, 5' GTC CCG CAG CGC AGA C (nucleotides 5889-5904), and *lac Z* reverse, 5' GCA GCG

TTG TTG CAG TGC (nucleotides 6236-6253), which amplify a 364 bp region of the *E. coli lac Z* gene. The *E. coli* O157:H7 *eae* TaqMan® probe is: 5' (FAM) CAG GCT TCG TCA CAG TTG CAG GC (TAMRA or BHQ), which spans nucleotides 1146-1168 of Genbank Accession No. AF253561, and the primers are: JK11 forward, 5' GGC GAT TAC GCG AAA GAT ACC (nucleotides 1104-1124); SE2 reverse, 5' CGC CAC CAA TAC CTA AAC GG (nucleotides 1415-1434), which amplify a 330 bp region of the *eae* gene.

PCR reactions were done in 50 μl volumes, containing 1 U *Taq* polymerase (Life Technologies, Gaithersburg, MD), 200 μM dATP, dGTP, dCTP, and dTTP, 1.5 mM $MgCl_2$, 5 μl 10X PCR buffer and 50 pmol of each primer; for TaqMan assays, 10X TaqMan "A" buffer (PE Biosystems, Foster City, CA) was substituted for regular 10 X PCR buffer and 10 pmol of probe was added. Cycling parameters were: 95° C for 1 min, followed by 40 cycles of 95° C for 15 sec, 52° C for 30 sec, and 72° C for 1 min. Real-time PCRs were conducted on the ABI Prism® model 7700 Sequence Detector instrument.

For experiments in which *Salmonella typhimurium*, *Listeria monocytogenes*, and *Bacillus anthracis* Sterne were used, overnight broth cultures of the organisms were processed using Instagene matrix, as described above. Four μl aliquots of Instagene-extracted DNA were assayed by real-time PCR on the Stratagene Mx4000® (La Jolla, CA) using specially packaged, freeze-dried reagents provided by Idaho Technology, Inc. (Salt Lake City, UT). The primers and probe sequences of the freeze-dried reagents are proprietary (for further information on these reagents, readers are referred to www.idahotech.com). Briefly, MBG water (40 μl) was added to each vial containing the freeze-dried reagents, gently agitated to dissolve the reagents, and then 20μl aliquots (i.e., two reactions per vial), were pipetted into 0.2 ml thermal cycling tubes. Bacterial DNA was added to the tubes and real time PCR performed for 1 min at 95° C, followed by 45 cycles of 95° C for 15 sec, and 60° C for 30 sec, with filters for LC Red 640 dye used to monitor fluorescence.

1.2 Extraction of DNA from *Cryptosporidium parvum* oocysts.

Cryptosporidium parvum oocysts (two to eight weeks of age) of the Beltsville strain were recovered from the diarrhea and manure of experimentally infected calves according to the procedure of Fayer et al. (2000).

Enumeration of oocysts was accomplished by pipetting 10 μl of the manure-derived total oocyst suspension onto a Neubauer hemacytometer and observing them under differential interference microscopy. A single count consisted of the mean of four different counts at the outer 4 x 4 grid of the hemacytometer. Counts from the hemacytometer method were used to prepare stock concentrations of oocysts at 1 x 10^6 oocysts per ml. Dilutions from these 1 x 10^6 oocysts per ml stocks were used for spiking experiments (results from a series of enumerations done on one such dilution derived from a 1 x 10^6 oocysts per ml stock are given in Table 1, below).

To count oocysts recovered from continuous flow centrifugation experiments, as well as to monitor the numbers of oocysts in dilutions made from the stock concentration for extraction experiments, 1 μl of a 15 - 100 μl total oocyst suspension was mixed with 1 μl of MeriFluor® immunofluorescence reagent (Meridian Diagnostics, Cincinnati, OH) and pipetted onto each well of a 3-well ring microscope slide (Cell-line™, Eerie Scientific, Portsmouth, NH). The slides were examined via IFA microscopy by the same two individuals throughout the project to ensure consistency in oocyst identification and enumeration.

Prior to DNA extraction, oocysts were lysed by freeze-thawing in a methanol dry-ice bath, or a -70° C freezer, and heat block / water bath. Initially we performed 5 -10 freeze-thawing cycles, but this was later reduced to 2 when microscopic examination of oocysts prestained with MeriFluor reagent indicated that the 100% were lysed with just 2 cycles, provided the lysis volume was 30 μl or less.

All attempts to reproduce the rapid DNA extraction protocol described by Hallier-Soulier and Guillot (2000) were unsuccessful; therefore, we used Instagene matrix, with the same protocol as for bacteria: following freeze-thawing, the lysed oocyst samples (30 μl), and an extraction control of MBG water, were added to 200 μl of Instagene and then incubated at 56° C for 15 minutes, followed by another heating step at 100° C for 8 minutes. The solution was then centrifuged (13,000 rpm, or approximately 13.4 x g , for 5 min) to pellet the Instagene resin, and 10 μl of supernatant (20 μl was used when under 100 oocysts were being extracted) was used as template for PCR.

In addition to the Instagene matrix, both the Xtra Amp tubes and PrepMan reagents were evaluated for rapid DNA extraction from oocysts; the protocols were the same as for the bacterial samples, save for the inclusion of a freeze-thawing lysis step prior to extraction.

1.3 Extraction of DNA from *Giardia lamblia* cysts.

Giardia lamblia cysts were obtained from the feces and diarrhea of experimentally infected calves housed in facilities at the USDA-ARS laboratories in Beltsville, MD using procedures similar to those described for isolation of *C. parvum* oocysts (Fayer et al., 2000). Enumeration of *Giardia* cysts was accomplished using the hemacytometer and immunofluorescence methods described above using the MeriFluor *Giardia* IFA reagents. After initial attempts to lyse *Giardia* cysts using freeze-thawing failed, a Bio101 FastPrep™ instrument was used to lyse the cysts. Briefly, approximately 200 µl of 0.1mm zirconia beads (BioSpec Products, Bartlesville, OK) and up to 1000 µl of water containing cysts were placed in a 2 ml, screw-capped plastic tube and subjected to homogenization for three, 45-second cycles at the FastPrep intensity setting of 5.0. The 2.0 ml tube was centrifuged at 13.4 x *g* for 3 min to pellet the zirconia beads and up to 30 µl of supernatant was subjected to DNA extraction using the Instagene matrix.

Another method was investigated for rapid lysis of *Giardia* cysts: using a Vortex Genie® 2 vortexer (made for Fisher Scientific by Scientific Industries, Inc. Bohemia, NY) and a 2 ml tube holder assembly that can be substituted for the rubber pad usually installed on the Vortex Genie 2. Cysts were placed into screw-cap, 2 ml tubes containing zirconia beads as described above and vortexed at maximum setting for 5 minutes. Ten µl aliquots of treated cysts were removed and examined for evidence of cysts lysis using a *Giardia* IFA commercial kit (MeriFluor®, Meridian Diagnostics, Cincinnati, OH).

1. 4 Recovery of *Cryptosporidium parvum* oocysts and *Giardia lamblia* cysts by continuous flow centrifugation (CFC).

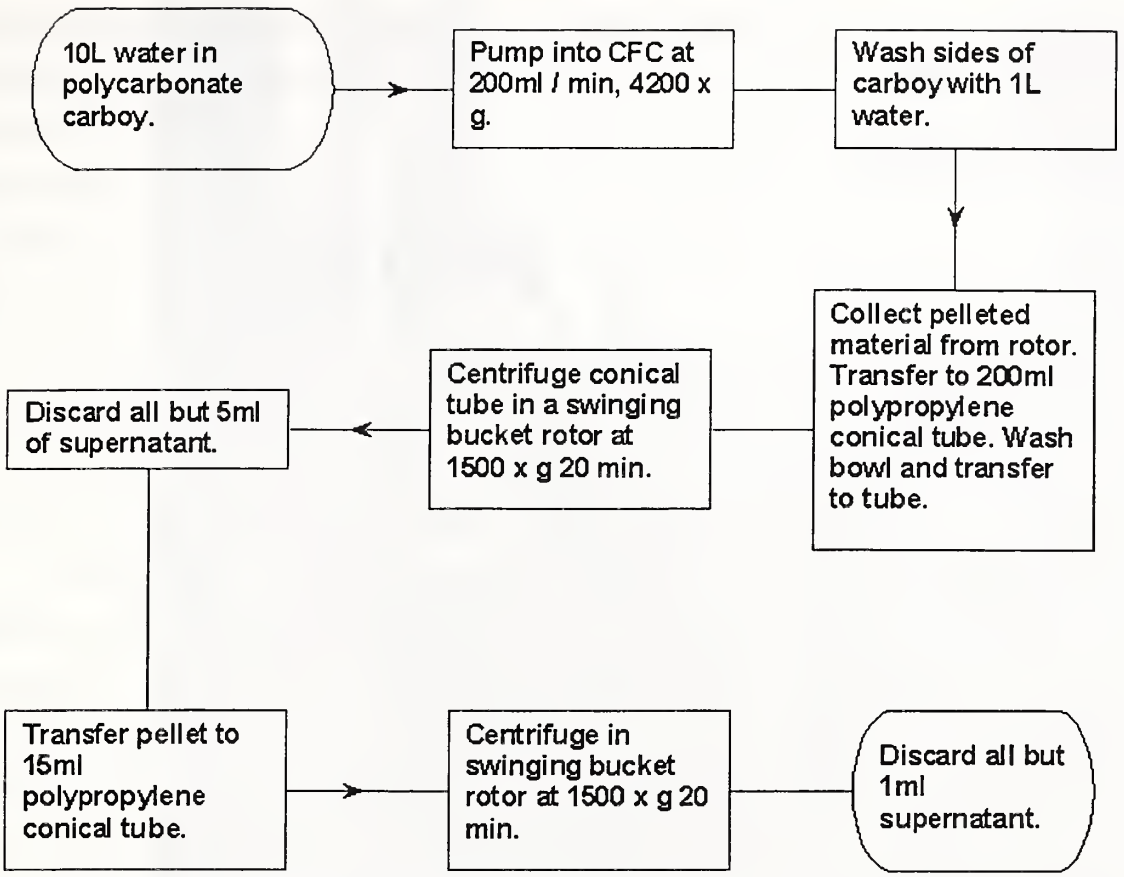


Figure 1.1. Flowchart of the continuous flow centrifugation (CFC) process.

Water samples were obtained from the Washington Suburban Sanitary Commission facility in Burtonsville, Maryland and later from Beaver Dam Creek, located in Beltsville, Maryland, and transported to the laboratory in 20 L plastic carboys. The setup for the CFC is shown in Figure 1.2. Oocysts and cysts were first spiked into 50 ml of sample water in a conical centrifuge tube, vortexed, and then deposited into a clean carboy containing the remaining 9.95 L of water. The carboy was shaken and then the contents pumped, using a MasterFLex™ peristaltic pump (Cole-Parmer, IL), through a No. 11 Model continuous flow centrifuge (Lavin, Hataboro, PA) at a rate of 200 ml per minute, at a speed of 7140 rpm (4200 x *g*). After passage of the entire 10 L of oocyst spiked source water, 1 L of tap water was added to the carboy, shaken to clean the sides and top of the carboy, and pumped through the centrifuge. Pelleted material (approximately 130 ml) was collected from the bowl of the CFC and transferred to a 200 ml polypropylene conical centrifuge tube. The bowl was then washed with approximately 30 ml of water which was added to the 200 ml conical tube; the combined eluate and wash volume of 160 ml was brought up to 200 ml with sterile water and the tube was

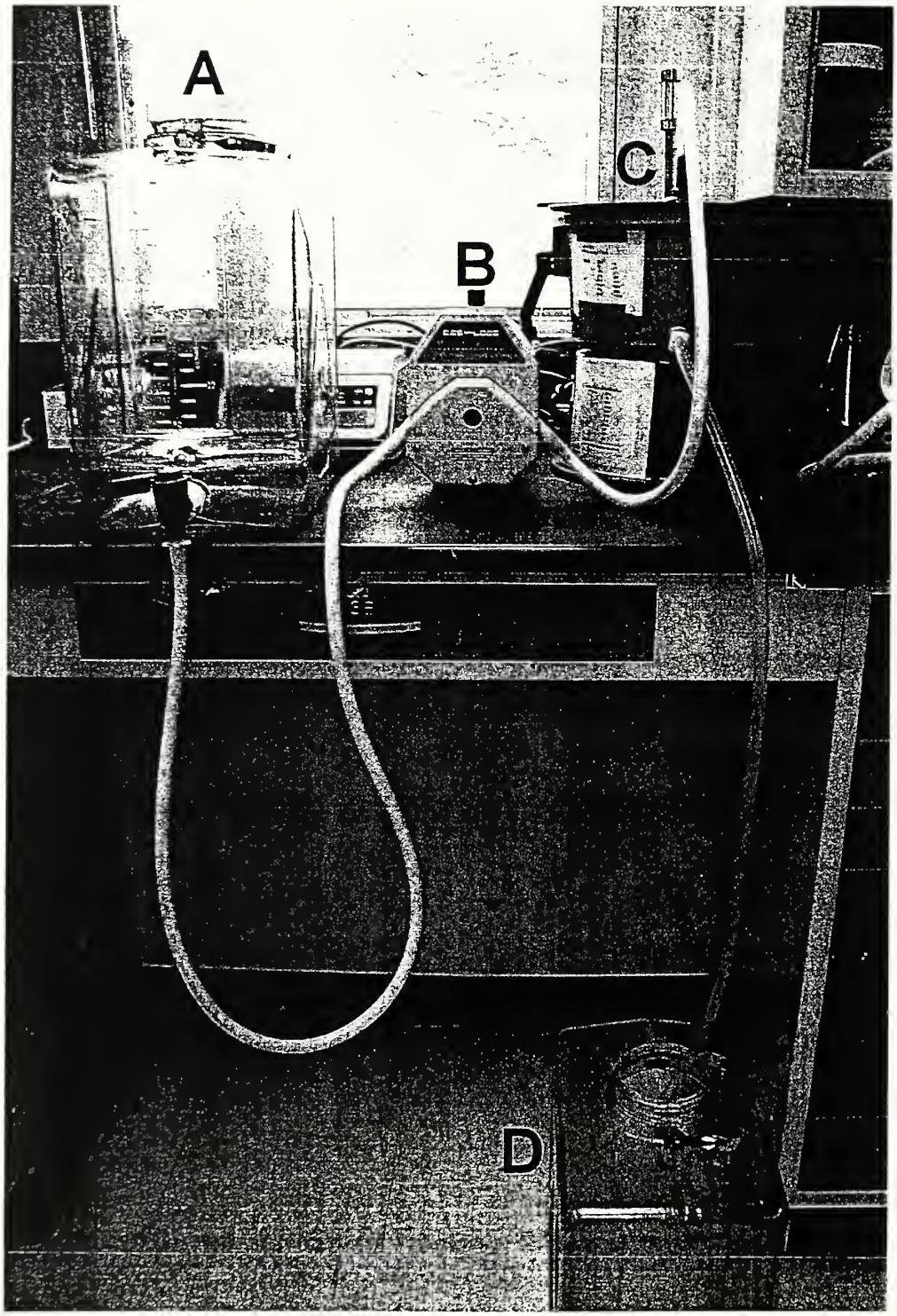


Figure 1.2. Setup for continuous flow centrifugation (CFC) recovery of *Cryptosporidium parvum* oocysts from spiked source water samples. A 10 L volume of source water in a plastic carboy (A) is pumped via a peristaltic pump (B) into a Lavin (Hataboro, PA) CFC (C) at a rate of 200 ml per minute. Overflow from the rotor is discarded in another carboy (D).

centrifuged in a Sorvall RC-2B ultracentrifuge (Kendro, Asheville, NC) with a swinging bucket rotor at 2800 RPM (1500 x *g*) for 20 min. All but approximately 5 ml of supernatant was aspirated and discarded. The pellet was then resuspended in the 5 ml of supernatant and transferred to a 15 ml polypropylene conical tube; 10 ml of sterile water were used to rinse the 200 ml tube, and this rinse then added to the 15 ml tube, which was then centrifuged in the swinging bucket rotor at 1500 x *g* for 20 min. Fourteen ml of supernatant was aspirated and discarded. For those samples undergoing IMS, the final recovery volume of approximately 1 ml was brought up to 10 ml with sterile water and subjected to IMS, using the Dynal Dynabeads® anti-*Cryptosporidium* kit (Lake Success, NY) and the Aureon™ anti-*Giardia* IMS kit (ImmTech, Inc. New Windsor, MD), according to the manufacturer's instructions, except that bead incubation was performed overnight rather than for 1 hr.

An unspiked control, consisting of either 10 L tap water or 10 L of source water, was performed to monitor for false-positive results due to the recovery of oocysts and cysts retained in the CFC apparatus.

For samples into which both *Giardia* cysts and *Cryptosporidium* oocysts were spiked, the first overnight IMS incubation was done using the Aureon anti-*Giardia* kit. The 10 ml, post-bead capture sample was then subjected to overnight incubation using the Dynal anti-*Cryptosporidium* kit, with 1 ml each of Buffer A and Buffer B simply being added to the sample, along with the required 100 μl of anti-*Cryptosporidium* Dynalbeads.

1.5 Detection of oocysts and cysts using PCR.

A variety of *C. parvum* genes were used as targets for PCR: Cp11, Cp41, 18S rRNA, and TRAP C-1. There were two reasons for this decision: first, rotating between different gene targets reduced the likelihood of contamination and false-positive PCR results, a potential problem when nested assays are performed; secondly, in our experience, those PCRs generating smaller-sized amplicons can be more sensitive, particularly for low copy number templates; and thirdly, the TRAP C-1 and 18S rRNA primers have been widely used by different laboratories and are arguably "standards" for the field of *C. parvum* detection.

For the Cp11 gene (Fayer et al., 1999; Genbank Accession No. AF124243), the outer primers were P5, 5' AAC ATC CAT CGA GTT TAG TA and P6, 5' GCA AGA GCG CAT TGG TGA AT; the inner primers were Cp11F, 5' GTC TAG AAC CGT TAC TGT TAC TGG and Cp11R, 5' CAA

CTC CTG GAA GCA TCT TAA CAG. The 18S rRNA primers were AL 1687 / 1691 and AL 3032 / 1598, which amplify 1.3 Kb and 0.83 Kb segments of the *C. parvum* 18S rRNA gene, respectively (Xiao et al., 1999). The Cp41 gene (Jenkins et al., 1999; Genbank Accession No. AF144621) outer forward primer was Cp41OF: 5' GAG GAG ATG GAC TAT TCT AGG; outer reverse primer Cp41OR, 5' GCA ACA GTA GTA AGA GTG GTA; inner forward primer Cp41 IF, 5' TGT ATG AAT TGG ATA TAT TAT TA; and inner reverse primer Cp41 IR, 5' GTA AAA GCA ACA CCA TTA CTA. The TRAP C-1 primers Cp.Z, Cp.W, and Cp.E were used according to the protocol of Spano et al. (1998); Cp.E and Cp.Z served as outer primers, and Cp.E and Cp.W as inner primers.

PCR reactions were done in 50 μl volumes, containing 1 U Taq polymerase (Life Technologies, Gaithersburg, MD), 200 uM each dNTP, 1.5 mM $MgCl_2$, 5 μl 10X PCR buffer and 50 pmol of each primer; for TaqMan assays, 10X TaqMan "A" buffer (ABI, Foster City, CA) was substituted for regular 10 X PCR buffer and 10 pmol of probe was added. For the *C. parvum* Cp41 gene nested PCR, cycling conditions were: 95° C for 1 min, followed by 35 cycles of 95° C for 15 sec, 50° C for 30 sec, and 60° C for 1 min. The PCR cycling conditions for the Cp11, 18S rRNA and TRAP C-1 assays followed the published protocols of Fayer et al. (1999), Xiao et al. (1999) and Spano et al. (1998), respectively.

For detection of *Giardia lamblia*, unpublished nested PCR primers targeting the triose phosphate isomerase (TPI) gene, designed by I. Sulaiman and L. Xiao, Centers for Disease Control, Atlanta, GA, were used. The outer primers are: AL 3543 5' AAA TIA TGC CTG CTC GTC G and AL 3544 5' CCC TTC ATC GGI GGT AAC TT. The inner primers are: AL 3545 5' GTG GCC ACC ACI CCC GTG CC and AL 3546 5' CAA ACC TTI TCC GCA AAC C. The expected size of the nested PCR product is approximately 500 bp, depending on the genotype. Quantities and concentrations of PCR reagents were similar to those used for *C. parvum* and the thermal cycling profile was 2 min, 95° C, followed by 40 cycles of 15 s at 95° C, 1.5 min at 55°C, and 2 min at 72° C.

1.6 Evaluation of different PCR primers for detection of 10 or fewer oocysts.

Coded samples consisting of 10 μl of MBG water containing 10, 5, 1, and zero oocysts were kindly provided by Greg Sturbaum and Charles Sterling, University of Arizona. The oocysts had been individually measured using a micromanipulator (Sturbaum et al., 2001). Prior to use, samples were subjected to 3 freeze-thawing cycles using a -70° C freezer and a 56° C water bath; those destined for

RT-PCR had 20 *U* of RNAse inhibitor (Perkin Elmer, Foster City, CA) added to the tube. The entire 10 μl volume of the samples was assayed via real time PCR on the Stratagene Mx4000 (La Jolla, CA) using the inner Cp11 primers (Fayer et al., 1999), the TRAP C-1 inner primers CpE and CpW (Spano et al., 1998) and the cphsp2423F and cphsp2764R (hsp70) primer pair (Rochelle et al., 1997; DiGiovanni et al., 1999). All samples received the same master mix, consisting of 200 uM each dNTP, 1.5 mM $MgCl_2$, 2.0 *U* AmpliTaq polymerase (Perkin Elmer, Foster City, CA), 5 μl SYBR™ Green 10X buffer (Perkin Elmer, Foster City, CA) and 50 pmol each primer. The 50 μl reactions were cycled for 2 min at 94° C, followed by 40 cycles of 94° C for 30 sec, 55°C for 30 sec, and 72° C for 1.5 mins.

For single tube RT-PCR, the Invitrogen Superscript™ reagent (Gaithersburg, MD) was used according to the manufacturer's instructions. Ten μl of water containing 0, 1, 5, and 10 oocysts were used as template, with 50 pmol each primer; the reaction conditions were: 42°C for 30 mins, 94° C for 2 mins, followed by 40 cycles of 94° C for 30 sec, 55°C for 30 sec, and 72° C for 1.5 mins.

1.7 Culture of MDBK cells, RNA extraction and detection of bovine enterovirus (BEV) RNA.

Bovine enterovirus type 2 (strain PS 87) was obtained from the American Type Culture Collection (Manassas, VA, USA). The virus was propagated in Madin Darby bovine kidney (MDBK) cells grown in Earle's minimal essential medium (MEM) containing 5% fetal calf serum and antibiotics. Cells were infected at the rate of 1 PFU/cell and the supernatant containing viral progeny was harvested after 24-36 h, when cytopathic effects (CPE) were observed in most (>80%) of the cell monolayer. The supernatant containing the virus was clarified by centrifugation at 5000 x *g* for 15 min at 4° C. The virus stock was titered in MDBK cells and stored at -70° C until used.

RNA extraction was performed on 140 μl of water eluate, or cell culture supernatant, using the Qiagen™ QIAamp® Viral RNA Mini kit (Valencia, CA) according to the manufacturer's instructions, except that RNA was eluted from the Qiagen columns in 60 μl of buffer (Ley et al., 2002).

The Xtra Amp RNA kit was also evaluated for RNA extraction from both MDBK cells and purified BEV virions. The MDBK cells were washed with PBS to remove traces of culture media (which can interfere with the nucleic acid-binding matrix in the Xtra Amp tube) and resuspended in PBS; multiple 50 μl aliquots of cells were added to the Xtra Amp tube containing 50 μl of lysis buffer. The combined volumes were mixed by pipetting, incubated at room temperature for 10 minutes, then

discarded. The tubes were twice washed with 175 μl of Wash Buffer, then dried with laboratory tissue paper and stored at 4° C until used.

RT-PCR was conducted on total RNA (approximately 100 - 250 ng, depending on the origin of the sample, and the perceived purity of the RNA) using the primer set for the 5' untranslated region for enteroviruses described by Schwab et al. (1995). The Invitrogen / Life Technologies Superscript® (Gaithersburg, MD) one-step RT-PCR reagent was used according to the manufacturer's instructions; cycling conditions were 42° C (this was later raised to 48° C and then to 50° C) for 30 min, followed by 94° C for 2 min, then 40 cycles of 94° C for 15 s, 48° C (later raised to 55° C) for 30 s, and 60° C (later raised to 72° C) for 1 min (Ley et al., 2002). The Ambion HAV Armored RNA™ (Austin, TX) internal control standard and a TaqMan GAPDH RT-PCR kit from ABI were used as controls for the quality of extracted RNA and the efficiency of the RT-PCR assay.

1.8 Extraction of nucleic acids using the ABI 6100 Nucleic Acid Prepstation.

Extraction of RNA followed the preloaded program for cultured cells. Briefly, MDBK cells were detached from a 175 cm^2 culture flask using trypsin-versene, transferred to a 15 ml conical centrifuge tube and centrifuged at 3,500 x *g* to pellet the cells. All but 1 ml of supernatant was discarded and the cells resuspended. Two hundred μl aliquots of the cell suspension were placed in a 1.5 ml microcentrifuge tube with 300 μl of RNA Lysis Buffer 1 and incubated for 10 min at room temperature. The entire sample was loaded into a well of the 96-well RNA tray, the tray placed in the ABI 6100 instrument, and subjected to the "20% vacuum" setting of the instrument for 2 min. Successive steps involved the addition of 300 - 500 μl of Wash Buffers 1 and 2 (2 washes with each) for 2 min at 20% vacuum, followed by a pre-elution vacuum step of 3 min with 90% vacuum. Following touch-off, elution was performed in 60 μl of RNA elution solution at 2 min, 20% vacuum.

The yield and quantity of RNA obtained from the ABI 6100 instrument, as well as that obtained using the Qiagen Viral RNA kit, was evaluated using a Beckman Coulter DU-640 model spectrophotometer (Somerset, NJ) and 50 μl microcuvette.

RESULTS

2.1 Rapid extraction of DNA from bacterial cultures.

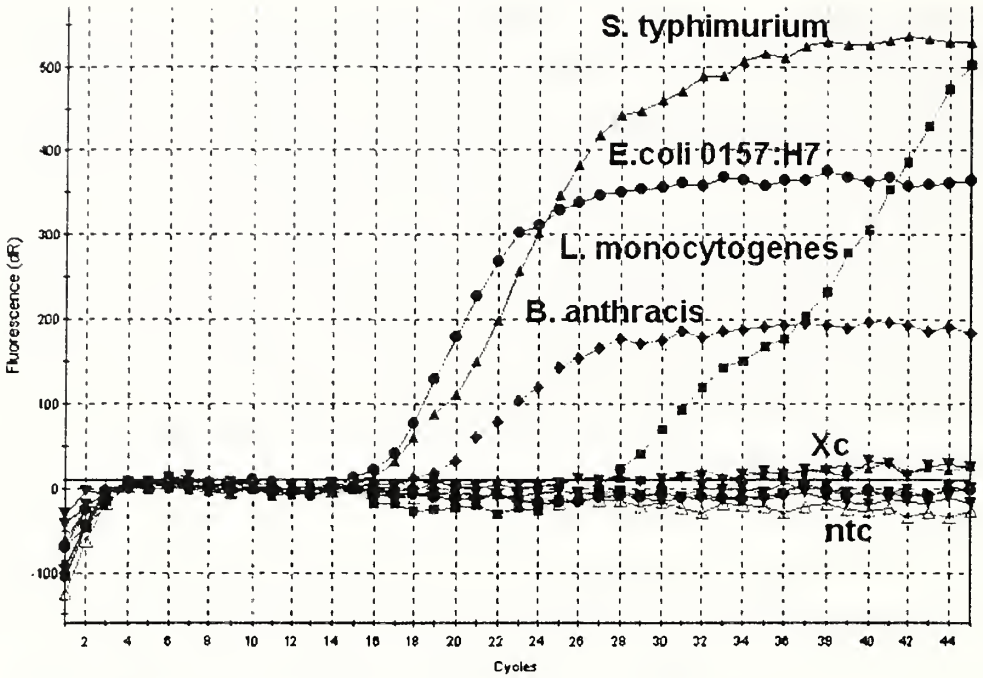
The Isocode paper, Instagene matrix, PrepMan and Xtra Amp tubes all provided PCR-quality DNA from overnight broth cultures of *E. coli* O157:H7, and from colonies on an agar plate (Higgins et al., 2001). The best yields of DNA, as determined by fluorescence obtained with real time, probe-based PCR, were generated by the Isocode paper and the Instagene matrix. The Xtra Amp tube method was the fastest and easiest method and required only a pipettor. The other three methods required, in addition to pipettors, a vortexer, a heat block and a small, countertop centrifuge. The price for the Xtra Amp tubes was approximately $1.80 per sample; for the other methods, the per-sample price was considerably lower, for example, under 50¢ for the Isocode paper. All methods allowed as many as 10 samples to be processed in under 1 hr.

Because it also provided satisfactory results with oocysts and cysts, was affordable, and relatively easy to use, we selected Instagene matrix as the rapid extraction reagent of choice and used it regularly for the duration of the Project. Instagene matrix-extracted DNA was suitable for use on a variety of commercial, real time, fluorescent probe-monitoring thermal cycler instruments, including the Cepheid Smartcycler® (Sunnyvale, CA), Idaho Technology Inc.'s RAPID™ (Salt Lake City, UT), the ABI Prism® 7700 Model Sequence Detector (Foster City, CA), and the Stratagene Mx4000 (La Jolla, CA) (data not shown).

Experiments conducted on other bacterial species, including gram positive ones, indicated that the Instagene matrix can successfully provide PCR-quality DNA from *Listeria monocytogenes*, *Salmonella typhimurium* and *Bacillus anthracis* Sterne strain, as well as *E. coli* O157:H7 (Figure 2.1A).

The utility of Instagene matrix for detecting DNA from *E. coli* O157:H7 cells spiked into a high number of nonpathogenic *E. coli* cells was investigated and data from one of several replicates is shown in Figure 2.1B. Using PCR primers to the *eae* gene, 10^4 *E. coli* O157:H7 per ml, against a background of 10^8 cells of nonpathogenic *E. coli* 11775, could be detected. This is equivalent to approximately 25 cells per 50 μl PCR reaction. It should be noted that this detection limit was achievable only when a more expensive formulation of *Taq* polymerase ["Platinum® *Taq* ", Invitrogen, Gaithersburg, MD], and bovine serum albumin, at 250 μg / ml, were added to the reaction; using regular *Taq* polymerase and no BSA resulted in a detection limit of only 10^6 *E. coli* O157:H7 cells per ml.

A


S. typhimurium
E.coli 0157:H7
L. monocytogenes
B. anthracis
Xc
ntc
Fluorescence (dR)
Cycles

B


500
300
L
10^6
10^5
10^4
10^3
10^2
10^1
10^0
Xc
ntc

C

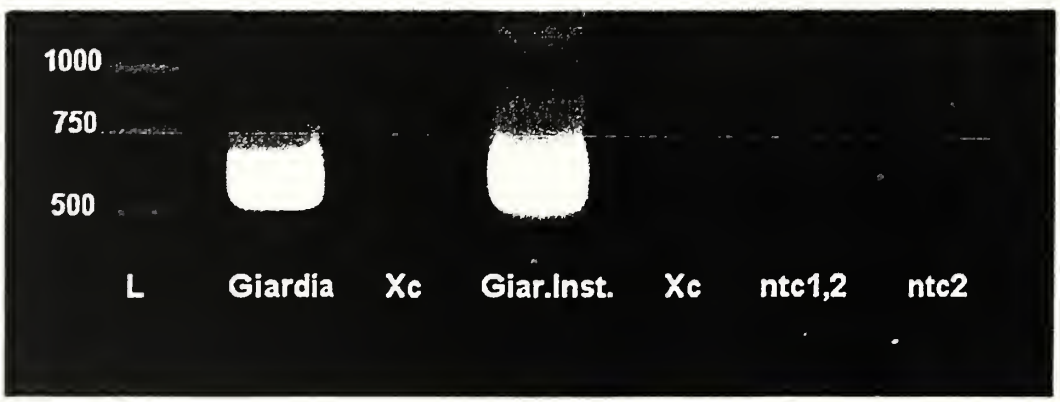

1000
750
500
L
Giardia
Xc
Giar.Inst.
Xc
ntc1,2
ntc2

Figure 2.1. Panel A: Real time amplification plots for four bacterial species (*Salmonella typhimurium, E. coli* O157:H7, *Listeria monocytogenes*, and *Bacillus anthracis* Sterne) using DNA extracted from overnight broth cultures using Instagene® matrix. Cycle number is plotted on the X axis and fluorescence on the y axis. No template control (ntc) and extraction control (Xc) plots are indicated. Panel B: Results of *eae* gene PCR conducted on various dilutions of *E. coli* O157:H7 cells spiked into a constant quantity of nonpathogenic *E. coli* 11775 cells in 1 ml. DNA was extracted from the dilutions using Instagene matrix. The quantities of *E. coli* O157:H7 cells per ml are indicated below the corresponding lanes. Lane L: DNA ladder, with rung sizes indicated; lane Xc: extraction control; lane ntc: no template control. Panel C: Results of nested PCR for the triose phosphate isomerase gene performed on DNA extracted from *Giardia lamblia* cyst lysate using phenol choloform isolamyl alcohol (lane "Giardia") and Instagene matrix (lane "Giar.Inst"). Lane L: DNA ladder, with rung sizes indicated; lanes Xc: respective extraction controls; lanes ntc1,2 and ntc2: primary and secondary no template controls, respectively.

2.2 Extraction of DNA from purified *C. parvum* oocysts and *G. lamblia* cysts.

Over 30 experiments were conducted in which various quantities of *C. parvum* oocysts were inoculated into 30 μl volumes of sterile water, subjected to several freeze-thawing cycles, and then DNA extraction using Instagene matrix. PCR assays using the Cp41, TRAP C-1, Cp11 and 18S rRNA gene primers were used on the resultant DNA. As described in Higgins et al. (2001), Instagene-extracted DNA was suitable for PCR; however, detection of small (under 100) numbers of oocysts required the use of nested PCR. Using nested PCR, it was possible to detect DNA extracted from as few as 3 oocysts (Higgins et al, 2001).

In contrast to *C. parvum* oocysts, *Giardia* cysts were refractory to lysis via the freeze-thawing method, even when liquid nitrogen was used in place of a methanol-dry ice bath. Consequently, the FastPrep instrument was used to lyse the cysts. This lysate was then subjected to DNA extraction with Instagene matrix, followed by PCR for the triose phosphate isomerase (TPI) gene. Results indicated that Instagene-derived DNA was comparable to phenol-chloroform-isoamyl alcohol extracted DNA in terms of PCR product yield (Figure 2.1C).

Because the FastPrep instrument may represent a substantial financial outlay for some water quality testing laboratories, an alternative method using the commercially available Vortex Genie 2 platform and special tube rack was evaluated. The same 2.0 ml screw-cap tube and zirconia beads were used as for the FastPrep machine, with the Vortex Genie bead beat time set to 5 min. Microscopic evaluation of beaten cysts (approximately 10^4) from 3 replicates revealed that at best, 25% of the cysts were fragmented, as determined by MeriFluor IFA staining patterns. The use of two

of the "ceramic spheres" provided in the FastPrep DNA extraction kit, in conjunction with the zirconia beads, improved the lysis rate to 80%.

2.3 Recovery of *C. parvum* oocysts and *G. lamblia* cysts from spiked source water sample using CFC.

Because of the importance of accurate enumeration of oocysts used in spiking experiments and the potential defects inherent in using a dilution method to determine oocyst quantities, an example of the results obtained from ten, 10-sample counts performed on one dilution used in a CFC spiking experiment (derived from a stock solution of 1×10^6 oocysts per ml) is shown in Table 2.1. Here, a spiking dose containing approximately 100 oocysts in 10 μl was being used. As is evident from Table 2.1, mean oocyst numbers (per 10 μl) were 97.3 with a range of 85 - 111; the coefficient of variation was 8.0. One interpretation of these results would indicate that the spiking doses used in the CFC experiments are reasonably accurate and that recoveries are not inflated due to the administration of larger doses of oocysts than predicted.

Table 2.1. Enumeration of *Cryptosporidium parvum* oocysts used in spiking experiments.

Rep#	Well #										Total
	1	2	3	4	5	6	7	8	9	10	
1	11	16	9	11	2	5	10	8	9	16	97
2	5	9	6	12	8	4	8	13	9	13	87
3	11	10	9	9	9	9	4	13	15	9	98
4	10	10	7	5	11	9	8	11	15	18	104
5	13	11	10	8	8	8	10	14	7	8	97
6	8	14	10	11	15	14	9	8	10	12	111
7	13	10	15	10	9	10	8	7	9	8	99
8	9	14	11	2	9	8	9	11	9	3	85
9	8	8	9	6	13	9	16	11	12	12	104
10	11	10	14	6	3	13	7	7	10	10	91
										$\bar{x} = 97.3 \pm 8.0$	

Ten, 1µl aliquots of oocysts were examined using MeriFluor IFA staining with 400x magnification.

Twenty assays were performed in which 100 oocysts were spiked into 10 L volumes of source water from the Washington Suburban Sanitary Commission (WSSC) and oocysts were recovered using an overnight Dynal bead IMS step. Ten of the assays subsequently relied on IFA

microscopy to detect recovered oocysts, while the other 10 assays subsequently used nested PCR for the *Cryptosporidium parvum* Cp41 gene and the TRAP C-1 gene.

As shown in Table 2.2, of the 10 replicates using IFA microscopy, 9 successfully detected oocysts, although recoveries were low (average of 4.4 %). Nine of the 10 replicates subjected to DNA extraction with Instagene matrix, and nested TRAP C-1 gene and nested Cp41 gene PCRs, successfully amplified. Oocysts were not observed in one unspiked control subjected to IFA microscopy and PCR assays on 3 unspiked samples were also negative.

Table 2.2. Recoveries of *Cryptosporidium parvum* oocysts spiked into 10 L volumes of source water and isolated using continuous flow centrifugation and immunomagnetic separation.

Replicate	No. oocysts spiked	No. oocysts recovered*
Unspiked control	0	0
5.18.01	100	8
5.29.01	"	5
5.30.01	"	6
6.5.01	"	0
6.18.01	"	4
6.19.01	"	3
6.20.01	"	4
6.25.01	"	7
6.28.01	"	3
7.18.01	"	4
		$\bar{x} = 4.4 \pm 2.2$

* recovery determined by IFA microscopy of Dynal™ bead/oocyst pellet

Because the recovery rates of oocysts in the experiments using a spiking dose of only 1 x 10^2 oocysts were so low, we investigated whether the bead capture step was responsible for reduced recovery. A set of 3 experiments was conducted in which 10 L of unspiked source water was subjected to CFC and the resultant pellet of sediment (0.5 - 1.0 ml) was mixed with 10 ml of water before IMS with the Dynal kit. This 10 ml volume was spiked with 1 x 10^3 oocysts and incubated overnight, followed by separation of the oocyst-bead pellet and examination with IFA microscopy. Numbers of recovered oocysts were 153, 81, and 151 (15.3%, 8.1%, and 15.1%, respectively) indicating that substantial numbers of oocysts were not being captured by the beads. Given the extremely high turbidities of the assayed samples, these results are perhaps not

surprising. NTU readings on the pellet of sediment recovered by the CFC, when mixed with 10 ml of water and Dynal kit buffers for the initial IMS step, routinely exceeded 400, and it was often necessary to dilute the sample several times in order to obtain a reading from the turbidometer. For example, of 6 samples of WSSC source water-derived CFC pellet in 10 ml of water and Dynal kit buffers, the average NTU reading was 982 (± 37.7, range 916 -1014). This is in contrast to the NTU values for WSSC source water samples, which ranged from 1.1 to 3.9, except for one source water sample from WSSC that had a value of 22. pH values for source water ranged from 6.7 to 7.3.

The successful detection of a spiking dose of 100 oocysts per 10 L led the investigators to try recovery of 10 oocysts spiked into 10 L of source water. Because of convenience, water from the Beaver Dam Creek (BDC), located on the USDA Beltsville campus, was substituted for WSSC source water. This water had a greater turbidity than that of the WSSC water (for example, three replicates of water collected in February, 2002 had NTU values of 134, 135, and 133; samples collected in March 2002 had values of 26.0, 27.0, 26.4, and 25.1) and, because the sampling site is located downstream of a sizeable manure composting operation, the presence of cysts and oocysts in the water obviously cannot be ruled out.

Results are shown in Table 2.3. The mean recovery was 3.2 ± 3.2 oocysts; the range 1-12 oocysts, and with 10 of 12 assays (83%) positive. Based on our enumeration of the oocysts used to spike the samples, with a coefficient of variation of 10% or less, the recovery of 12 oocysts from the October 4, 2001 replicate is theoretically feasible, but we cannot rule out inflation of the recovery by oocysts already present in the sample. In an attempt to rule out counting of naturally occurring oocysts, we used prestained (with MeriFluor) oocysts to spike the 6 samples assayed from February 19-February 28, 2002; there were no recoveries from these samples. We were not sure if the prestaining regimen interfered with the binding of the antibody reagent in the Dynal kit (the identity of the antibodies and their epitopes are not divulged by Dynal and Meridian Diagnostics [maker of MeriFluor]) to the extent of hampering recovery; nor are we sure if the prestained antibody remained intact through the recovery process. When we discontinued the use of prestained oocysts for the 4 replicates conducted from March 7 - March 28, 2002, two replicates were positive, with 3 and 1 oocyst recovered.

Table 2.3. Recoveries of *C. parvum* oocysts from 10 L volumes of source water spiked with 10 oocysts.

Date	No. Recovered	Remarks
10.3.01	3	WSSC source water
10.4.01	12	
1.30.01	6	BDC source water
1.31.02	3	
2.1.02	1	
2.12.02	3	
2.14.02	4	
2.15.02	3	new batch of BDC source water
3.7.02	3	new batch of BDC source water
3.22.02	1	
3.26.02	0	
3.28.02	0	
10.10.01	0	unspiked tap water control
10.18.02	0	unspiked tap water control
2.5.02	1	unspiked control
4.02.02	0	unspiked control

SUMMARY STATISTICS: $x = 3.2 \pm 3.2$; range 1-12 oocysts; 10/12 assays (83%) positive

WSSC: Washington Suburban Sanitary Commission (Burtonsville, MD)
BDC: Beaver Dam Creek (Beltsville, MD)

Five of 7 replicates (February 12-March 28, 2002) receiving a spiking dose of 10 prestained *Giardia* cysts were positive, with recoveries of 1,1,1,1,and 2 cysts ($x = 0.8 \pm 0.7$).

As with the WSSC source water CFC pellets, turbidities of the corresponding BDC CFC pellets resuspended in 10 ml water and IMS buffers was very high (regularly in excess of 400 NTU) with mud and silt particles clearly visible.

2.4 Recovery of RNA from cultured cells and BEV.

In our hands, the Qiagen Viral RNA kit provided better yields of RNA than the ABI 6100 Nucleic Acid Prepstation from both uninfected MDBK cells and from infected MDBK cells, as determined by both spectrophotometry and RT-PCR. For example, Table 2.4 shows results for each method for four replicates; even allowing for the presence of contaminating DNA (the samples were not DNAsed prior to analysis) the yields obtained with the Qiagen method were substantially greater than that of the ABI 6100. When real time RT-PCR was conducted for the glyceraldehyde phosphate dehydrogenase (GAPDH) gene on this extracted RNA, fluorescence signals were substantially stronger for the Qiagen-derived samples as opposed to the ABI 6100 samples. When we attempted to improve RNA yield from the ABI 6100 by increasing the quantity of MDBK cells subjected to extraction, the filters on the RNA plate became clogged and it was impossible for the lysis and wash buffers to flow through them into the waste tray. When RT-PCR for BEV was conducted on RNA from infected MDBK cells, only Qiagen-extracted RNA allowed amplification.

Results of assays performed on viral RNA extracted from water samples associated with agricultural use, using the Qiagen Viral RNA kit, are provided in Ley et al. (2002).

We also examined the use of the Xtra Amp tube for RNA extraction from MDBK cells. When equivalent volumes of cells were extracted using this method and the Qiagen method, and the resultant RNA analyzed by real time RT-PCR for the GAPDH gene, the fluorescence signals from the Qiagen-derived RNA were substantially greater than that of the Xtra Amp tubes (Figure 2.4), indicating substantially greater RNA yield with these reagents.

Finally, the ABI 6100 was evaluated for extraction of DNA from bacterial cultures and colonies. The RNA plate was used in an attempt to reduce the cost associated with each extraction, since the DNA plate (which much be used in conjunction with the RNA plate for genomic DNA extraction from samples), costs another $100.00 per plate. We operated under the assumption that the ABI 6100-derived RNA would contain enough contaminating genomic DNA to allow for successful PCR. Overnight broth cultures of *L. monocytogenes*, *S. typhimurium*, *B. anthracis* Sterne, and *E. coli* 157:H7 (30 μl of the pellet from 1 ml culture) all yielded PCR-positive DNA. Loopfuls of *L. monocytogenes* from an agar plate were also successfully extracted and generated satisfactory amplification, save for one replicate of 6 (Figure 2.2B).

Table 2.4. RNA yield from bovine cells using the Qiagen Viral RNA kit (Nos. A-D)and the ABI 6100 automated nucleic acid extraction instrument (Nos. 1-4).

Sample ID	Abs 260 nm	260/280 ratio	μg / μl
A	0.69	2.8	0.139
B	0.29	3.3	0.059
C	0.67	2.9	0.134
D	0.52	3.1	0.105
1	0.09	1.8	0.018
2	0.09	1.8	0.019
3	0.07	1.7	0.015
4	0.06	1.6	0.012

Each replicate contained an equal volume of MDBK cells suspended in MEM with 5% FCS

Ten μl out of an eluate volume of 60 μl were assayed in a 50 μl microcuvette on a Beckman DU-640 model spectrophotometer

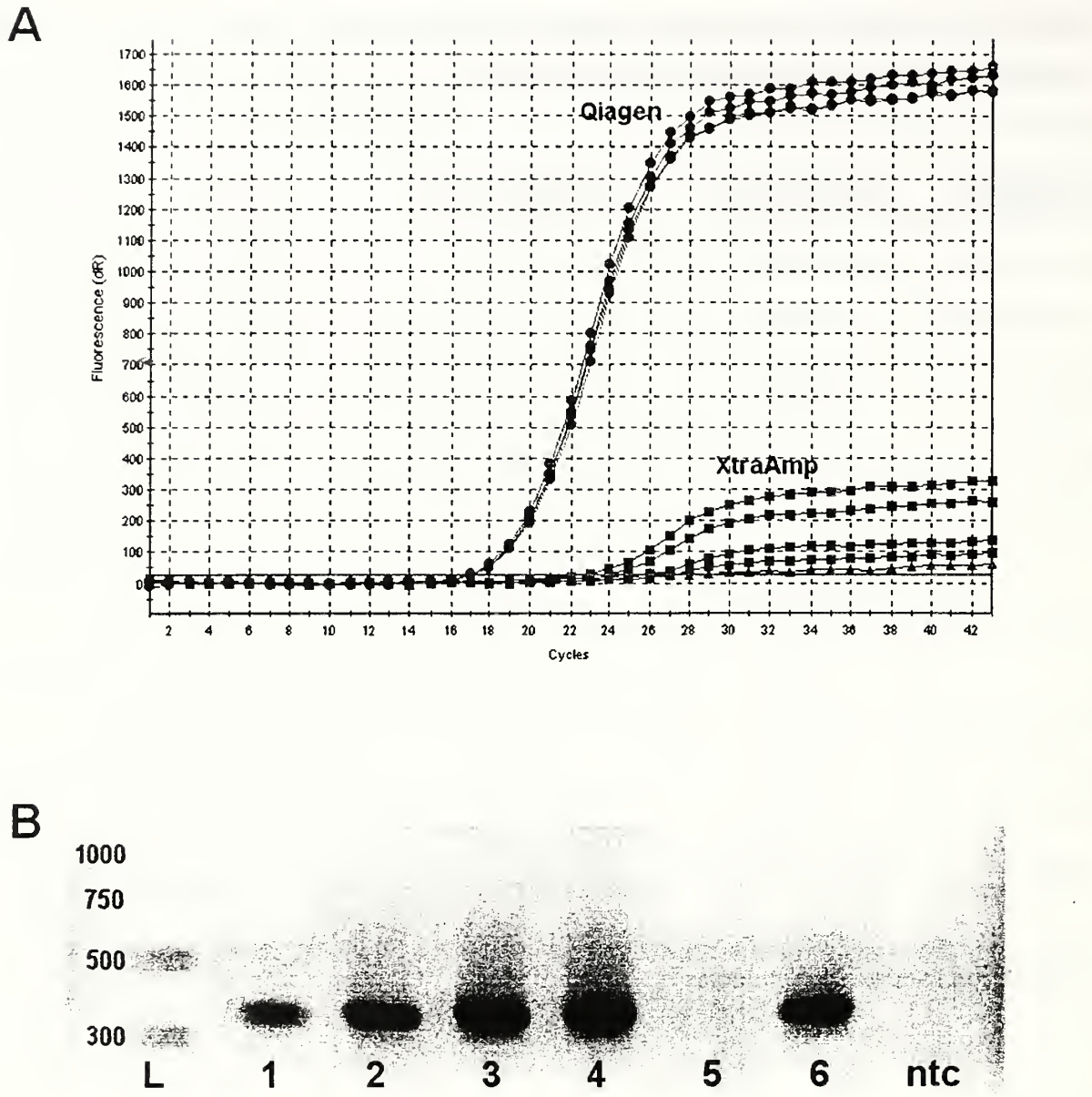


Figure 2.2. Panel A: Real time RT-PCR plots (labeled) for the GAPDH housekeeping gene conducted on RNA extracted from bovine cells using the Qiagen™ Viral RNA kit, and Xtra Amp RNA tubes. Cycle number is plotted on the X axis and fluorescence on the y axis. No template control plots are located under the threshold line (the black horizontal line at the bottom of the graph). Panel B: results of *iap* gene PCR conducted on DNA extracted from six individual agar plate colonies of *Listeria monocytogenes* using the ABI 6100 automated nucleic acid extraction instrument (lanes 1-6). Lane L: DNA ladder, with rung sizes indicated; lane ntc: no template control.

2.5 Evaluation of different PCR primers for detection of 10 or fewer oocysts.

Prior to evaluating small (10 or fewer) quantities of oocysts with three different primer sets, the effect of the shared reaction condition on the efficiency of each primer set was investigated by using DNA extracted from 1 x 10^6 oocysts as a positive control. As shown in Figure 2.3A, amplification plots for each primer pair were very similar; the mean threshold cycle (C_t) value for the TRAP C-1 primer set was 16.5 (+/- 0.8)(n = 3 replicates), for the hsp70 primer set 15.8 (+/- 0.1), and for the Cp11 primer set 15.5 (+/- 0.1). The slightly higher C_t value for the TRAP C-1 primer set reflects the increased size of this amplicon (approximately 500 bp compared to 330 for Cp11 and 361 for hsp70). We interpreted these results to indicate that all the primer sets were operating with equivalent efficiency under these reaction conditions and with these reagents. Consequently, each of the primer pairs were assayed using 10, 5, 1, and zero oocysts. As shown in Figure 2.3B, none of the samples amplified save the positive controls, and only the positive controls had SYBR Green fluorescence in excess of that of the zero oocyst and no template controls.

Since some investigators have reported detection of one oocyst in a packed pellet volume of 100 μl (equivalent to that recovered from 50 L of source water using a fiberglass depth cartridge filter with a minimal pore size of 1 μm; Kaucner and Stinear, 1998) using hsp70-derived primers in an RT-PCR assay, we tried using our hsp70 primers in a single tube RT-PCR to see if this improved sensitivity. Results indicated that 5 and 10 oocyst samples were clearly positive, with a very faint band discernable for the 1 oocyst sample (Figure 2.3C). However, when RNA extracted from six, 2 ml aliquots of Beaver Dam Creek (BDC) water was assayed using the hsp70 primers, strong amplification was observed for all 6 samples (Figure 2.3D).

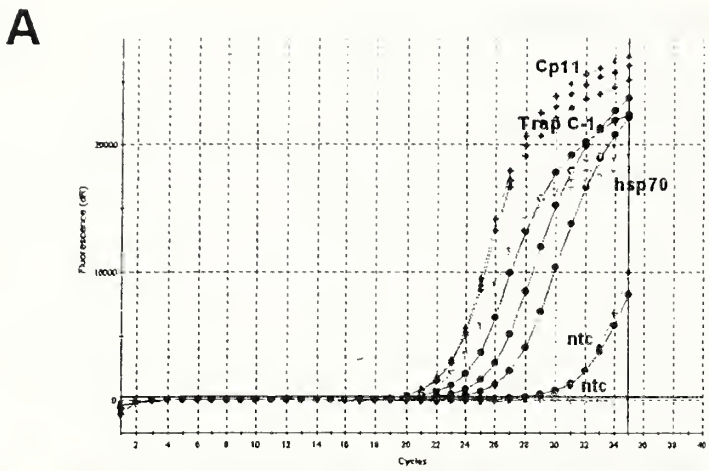


Figure 2.3. Panel A: Real time PCR plots for three different *Cryptosporidium parvum* primer pairs (Cp11, TRAP C-1, and hsp70) conducted on DNA equivalent to 1 x 10^6 oocysts per reaction. Three replicates were used for each primer pair. Cycle number is plotted on the X axis and fluorescence on the y axis, and no template control plots are located in the bottom right-

hand corner of the graph. Panel B: Results of PCR conducted on 0, 1, 5, and 10 purified oocysts of *C. parvum* using the Cp11, TRAP C-1, and hsp70 primers. For convenience, amplification products from the three separate reactions were electrophoresed on the same agarose gel. Lane L: DNA ladder with rung sizes indicated; lane ntc: no template control; lane pos: positive control DNA; note that the amplicons for the Cp11 (330 bp) and hsp70 (361 bp) are too close in size to be distinguished; the TRAP C-1 amplicon is approximately 500 bp in size. Panel C: Results of *C. parvum* hsp70 gene RT-PCR conducted on RNA and DNA extracted from 0, 1, 5, and 10 oocysts. Lane L: DNA ladder with rung sizes indicated; lane ntc: no template control. Panel D: Results of *C. parvum* hsp70 gene RT-PCR conducted on RNA and DNA extracted from 6, 2 ml aliquots of Beaver Dam Creek water (lanes 1-6). Lane L: DNA ladder with rung sizes indicated; lane ntc: no template control; lane +ctrl: positive control (*C. parvum* RNA).

DISCUSSION

3.1 Extraction of DNA from bacteria.

As detailed in Higgins et al. (2001), the four methods investigated: Xtra Amp tubes, Instagene matrix, Isocode paper, and PrepMan reagent all gave satisfactory results when extracted DNA was assayed by real time PCR for various *E. coli* genes. By virtue of being able to use a larger quantity of template (up to 20 μl), the strongest real time signals were associated with the Isocode paper-derived DNA. Each end user will of course want to bring their own needs and opinions into play when selecting a reagent for use in their laboratory, but we decided on Instagene matrix as offering the best combination of economy, ease of use, and effectiveness, and used it to extract DNA from other species of bacteria, including gram positive ones such as *Listeria*. The DNA extracted from these species was suitable for real time and conventional PCRs (Figure 2.1A). We conclude that Instagene matrix is a good choice for those investigators wanting a simple method to rapidly obtain PCR-quality DNA from variety of bacterial preparations, provided ancillary equipment, such as centrifuges, heat blocks, and a vortexer, are present. For laboratories lacking these, the Xtra Amp tube may be a more effective reagent.

In addition to Instagene matrix, we also evaluated the ability of an automated nucleic acid extraction instrument, the ABI 6100, to provide PCR-quality DNA and RNA from bacterial samples. As shown in Figure 2.4B, using the RNA plate in an attempt to reduce assay costs did succeed in yielding satisfactory DNA, at least from the majority of replicates. However, given the cost of the ABI

6100 (approximately $16,000, with 10-packs of plates and associated reagents costing well over $1,000), we feel it is uneconomical for use by water quality testing laboratories unless they are performing PCR on a very large number of samples (100 or more) per day.

Some of our bacterial PCRs (Figure 2.1A) were done using novel, freeze-dried real time PCR reagents (Idaho technology, Inc., Salt lake City, UT). Use of these reagents simply involves adding MBG water to the tube containing the pelleted PCR mix, and then transferring the reconstituted reagents to the PCR reaction vessel (in this case, the glass capillaries used by the Lightcycler [Roche, Indianapolis, IN] / RAPID [Idaho technology, Inc., Salt lake City, UT]). The shelf life of these freeze-dried reagents can be longer than 6 months. While freeze-dried PCR reagents (such as Pharmacia's "Ready To Go™ " beads) have historically been available, the Idaho Technology reagents include a positive control, preformatted primers and probe, and use identical cycling conditions, so that a number of different targets can be assayed on one PCR run. Currently the reagents use Lightcycler Red dyes which limits their use to the Lightcycler and RAPID real time PCR instruments (we were able to adapt them to the Stratagene Mx4000 instrument by using special filters), and cost $10.00 per reaction, two factors which probably make them unsuitable for widespread adoption by water quality laboratories. However, as other companies (Cepheid, Sunnyvale, CA) enter this market, it is reasonable to expect that costs for the freeze-dried reagents will decrease, and they will be adaptable to a greater variety (and price range) of real time instruments. In the scenarios we envision, where laboratories may want to test some of their routine bacterial cultures for the presence of important enteric pathogens, freeze-dried reagents may provide a user-friendly way to conduct molecular biology - based assays without the need to maintain stocks of *Taq* polymerase, dNTPs, positive control cultures, etc. It is anticipated that freeze-dried RT-PCR reagents will be available within the next year, which would allow for detection of some viral agents as well.

3.2 Extraction of DNA from *C. parvum* oocysts and *G. lamblia* cysts.

The impetus to use Instagene matrix to extract DNA from *C. parvum* oocysts and *Giardia* cysts came from a paper by Hallier-Soulier and Guilot (2000), who reported detection of as few as one oocyst in 20 L of source water using capsule filtration, immunomagnetic separation, DNA extraction with Chelex™ resin (Instagene matrix is a 6% solution of Chelex, marketed by Bio-Rad, Hercules, CA), and one round of 18S rRNA PCR. Despite numerous experiments, we were unable to reproduce

the sensitivity reported by these authors, even when assaying purified oocysts as opposed to oocysts recovered from spiked source water samples. The best sensitivity we could achieve was approximately 3 oocysts, spiked into a 50 ul reaction volume, using nested PCR. In our hands, detection of fewer than 100 oocysts required nested PCR, even when various primers, targeting different genes, were used.

To determine how well the Instagene method would apply to oocyst preparations more reflective of actual environmental samples, we recovered oocysts from spiked (n = 100 oocysts per 10 L) source water samples by the CFC / IMS procedure. The oocyst-Dynal™ bead pellet was subjected to freeze-thawing and DNA extraction with Instagene matrix, and then nested PCR for the TRAP C-1 (Spano et al., 1998) and Cp41 (Jenkins et al., 1999) genes. For each primer pair, 9 of 10 replicates were positive, indicating that the Instagene matrix could successfully extract DNA from these types of samples.

We also evaluated the utility of Instagene matrix to extract PCR quality DNA from cysts of *Giardia*. While we found that use of zirconia beads and a bead-beating apparatus was necessary to lyse the cysts, satisfactory extraction of DNA from the lysate was possible with Instagene matrix, offering improved extraction time and ease of use over the phenol-chloroform-isoamyl alcohol method (Figure 2.1C).

3.3 Continuous Flow Centrifugation (CFC) for recovery of oocysts and cysts from spiked source water samples.

As part of a project with the American Water Works Association Research Foundation (No. 2502, "Advancing *Cryptosporidium parvum* detection methodologies", M. Jenkins, USDA-ARS, Beltsville, MD, PI), we had begun investigation of CFC to recover oocysts from finished and source water samples (10L) from the Washington Suburban Sanitary Commission (WSSC) in Burtonsville, MD, and the Baltimore Bureau of Waste Water (BBWW), Baltimore, MD. Initial experiments utilized large spiking doses (1×10^5) of oocysts, to insure that enough were recovered to dispense for fluorescent in situ hybridization (FISH), cell culture PCR (ccPCR), nested PCR, and IFA microscopy. All of these methods were found to work well on recovered oocysts, indicating that the oocysts were not damaged or appreciably altered by the CFC procedure (we did notice that ccPCR occasionally generated positive results from cells inoculated with dead oocysts).

The next phase of the experiment focused on smaller spiking doses in source water, with IFA microscopy and nested PCR selected as the methods to measure oocyst recovery. Nine of 10 replicates had oocysts detected by IFA when the spiking dose was 100 oocysts in 10 L (Table 2.2). As mentioned above, another 10 replicates were assayed by nested PCR, with 9 of 10 positive. When the spiking dose was reduced to 10 oocysts in 10 L, 10 of 12 assays (83%) were positive, with a mean oocyst recovery of 3.2. We cannot be certain that the recoveries were inflated by oocysts already present in the samples, given that an unspiked control (Feb. 5, 2002) came up with 1 oocyst recovered. We tried to address this issue by spiking with prestained oocysts, but when six consecutive replicates came up negative we began to suspect that the MeriFluor antibody was interfering with that used by the Dynalbeads. This did not seem to be the case when the dedicated MeriFluor reagent was used to stain *Giardia* cysts and they were recovered using the Aureon IMS kit; we co-spiked 7 of the Beaver Dam Creek water samples (February 20-March 7, 2002) with 10 cysts of *Giardia*, and observed that 5 of 8 replicates were positive, indicating that the CFC method may be useful for detection of this pathogen. Zuckerman et al. (1999) also successfully used CFC to detect *Giardia* in river water samples, although they used a substantially higher spiking dose in a larger volume of water (1.1×10^4 cysts in 45 L).

We conclude that the CFC method deserves some consideration as an alternative to the capsule filtration method currently used by many water quality testing laboratories (i.e, USEPA Method 1623)(Matheson et al., 1998; Pezzana et al., 2000). It does require a higher initial investment: approximately $8,000 for the CFC, another $2,000 for the peristaltic pump; we assume that most laboratories will have a tabletop centrifuge for the latter concentration steps of this protocol, and will not need to purchase one. With each Gelman Envirochek™ capsule costing approximately $90.00, the CFC method will not be comparatively cost-effective until more than 100 of these capsules have been used. However, we believe the CFC method's greater ease of use may justify the increased set-up costs; also, the IMS and IFA reagents would be the same as for the 1623 method, and therefore not represent additional outlay (Swales and Wright, 2000).

3.4 Evaluation of different PCR primers for detection of 10 or fewer oocysts.

In light of the fact that most studies observe a concentration of *C. parvum* oocysts in surface waters of less than 1 oocyst per L (Smith and Rose, 1998), it is reasonable to postulate that any PCR performed on concentrated water samples will have very small number of oocysts (10 or fewer)

available for amplification. The question then becomes: which PCR assay is best suited to detect this small a quantity of oocysts? Obviously, the literature on this subject is sizeable; to cite just a few examples, some researchers can detect 10 or fewer oocysts with one round of PCR or RT-PCR (Kaucner and Stinear, 1998; Hallier-Soulier and Guillot, 2000; Lowery et al., 2000), while others report a nested PCR is required (Sturbaum et al., 2001; Jellison et al., 2002). When attempting to design detection protocols for use by the water industry, there is a reluctance to rely on nested PCR, because of the increased risk of contamination and false-positive results with this technique; therefore, a "one-round-only" PCR is preferred. With this preference in mind, we decided to assay three PCR primers in use in our lab, and by other investigators, to determine which one, if any, offered superior sensitivity. When we used the TRAP C-1 (Spano et al., 1998), Cp11 (Fayer et al., 1999) and hsp70 (Rochelle et al., 1997) primers on quantities of 0, 1, 5, and 10 oocysts measured by micromanipulator, none of the PCRs were positive. We then selected the TRAP C-1 and hsp70 primers for use in a one-tube RT-PCR reaction and observed positive results with the hsp70 primers for 5 and 10 oocysts, with a faint band for the 1oocyst sample (Figure 2.5C). It should be noted, however, that other investigators have found that hsp70-derived primers amplify non-*Cryptosporidium* organisms present in source water samples (Kaucner and Stinear, 1998), and while the cphsp2423 / cphsp2764 primer set was originally described as being specific for *C. parvum* (Rochelle et al., 1997), we found it amplified DNA/RNA from organisms present in 2 ml of creek water (Figure 2.3D). Given that the results of CFC experiments using BDC water, which indicate naturally occurring oocysts are very rare even in 10 L concentrates, we feel it is highly unlikely the amplification observed in these samples is due to the presence of *C. parvum* RNA or DNA. It may be possible to use oligonucleotide probes to confirm the identity of hsp70 amplicons; however, it is unclear if water testing laboratories will be willing to invest in the time and labor required to perform nonradioactive Southern Blot assays, nor if they are willing to purchase a real time thermal cycling instrument to conduct fluorogenic probe-based assays.

Also, the fact that we saw a very faint band for 1 oocyst, even when using purified oocysts directly in the PCR reaction, is in contrast to reports from other laboratories where 1 oocyst can be detected following recovery from large volumes of water (Hallier-Soulier and Guillot, 2000), or when spiked into 100 μl of filtrates from a large volume of source water (Kuacner and Stinear, 1998). Our results raise questions as to whether single oocysts can be reproducibly detected in these complex sample matrices. We intend to continue our evaluation of single tube, hsp70 RT-PCR in an effort to improve sensitivity and specificity; for example, raising the temperature at which reverse transcription

takes place, from 42° C to 50° C or higher, may improve the specificity by preventing hybridization of the primers to nontarget sequences.

3.5 Evaluation of different RNA extraction methods.

The ABI 6100 is marketed for high-throughput extraction of RNA and DNA from a variety of sample types, including cultured cells and blood. In our hands, its performance was disappointing; while RNA was successfully extracted from MDBK cells, the yield was substantially smaller than that obtained with the Qiagen Viral RNA kit (Table 2.4), and the results of real time RT-PCR for the GAPDH housekeeping gene were so poor that we did not focus further efforts of BEV RT-PCR on any 6100 -derived RNA samples. We also abandoned plans to try the ABI 6100 for RNA extraction from BEV virions spiked into source and finished water samples.

In an effort to justify the $16,000 spent on this instrument we tried extracting nucleic acids from bacterial cultures and colonies; the DNA yield with these samples was sufficient to successfully perform PCR (Figure 2.2B). If any water laboratories are interested in utilizing this instrument for this purpose, we would recommend using the RNA plates for DNA extraction, rather than spending extra money to get the dedicated DNA plates. But unless the laboratory plans to assay at least 100 or more samples a day, the ABI 6100 is uneconomical and we would advise the use of other methods. [With this said, the ABI 6100 is less expensive than other automated platforms for nucleic acid extraction, such as the MagNA Pure® system from Roche at >$80,000 and the Instant Genetics Autolyzer™ at >$50,000].

Since the Xtra Amp tube was the fastest and most user-friendly method for DNA extraction from bacteria, and was also useful for extracting DNA from lysed *C. parvum* oocysts, we investigated the new RNA Xtra Amp tube. The protocol is essentially the same as for DNA, and we found that the Xtra Amp tube did successfully extract RNA from MDBK cells, as determined by RT-PCR for the GAPDH gene (Figure 2.2A). However, the RNA yield was lower than that obtained with the Qiagen Viral RNA kit and potential end users will have to decide if the convenience of the Xtra Amp tube offsets the reduced RNA yield.

3.6 Conclusion.

Our approach to the project was guided by the consideration that molecular methods for coliform detection and enumeration are too expensive, and require more intensive training, than culture and biochemical-based methods currently employed in water testing laboratories. Perhaps most importantly, molecular methods are not accepted by regulatory agencies such as the US EPA. Therefore, we certainly do not envision molecular methods as supplanting those now in use, such as the Colilert™ technique. But we do see a role for molecular methods in providing important ancillary information to laboratory managers, in scenarios that may involve asking: is the *E. coli* in water sample X pathogenic (i.e., *E. coli* O157:H7)? Given that a bacterial culture generated from routine coliform testing will already be present in these laboratories - for example, a Colilert Quanti-Tray™-- we feel it entirely feasible for a sample to be removed from the Quanti-Tray, briefly centrifuged to pellet the bacteria, and processed for DNA extraction using one of the four methods investigated in this project. Less than 1 hr later the sample would be ready for PCR (which, if performed using freeze-dried reagents on a real time instrument, would bring the total time from DNA extraction to final results in under three hours for as many as 10 samples).

In the case of pathogenic, encysted protozoa, such as *Cryptosporidium*, the challenges in providing a rapid DNA extraction method for molecular-based detection are more formidable. First, the organisms must be recovered from a large volume of water, which in the case of source water, may contain potential PCR inhibitor(s). We evaluated the CFC technique for this purpose and found that it may offer some advantages over the conventional US EPA method 1623. Secondly, the oocysts must be lysed to release enough DNA for extraction; we found that freeze-thawing was suitable for this purpose. Third, DNA extraction proper should be as simple as possible and avoid the use of hazardous reagents such as phenol; as other investigators have noted, we found that Instagene matrix, a 6% solution of Chelex resin, was suitable for this purpose. Finally, the PCR assay should rely on one round of amplification, if possible, for successful detection. In our hands, we found a nested PCR was necessary to detect fewer than 100 purified oocysts, regardless of the choice of target gene: Cp11, Cp41, 18S rRNA, TRAP C-1, or hsp70. The latter gene target may offer the best chance for detection of as few as one oocyst when an RT-PCR reaction is conducted, provided specificity can be improved.

As part of the project we evaluated a new, automated instrument, the ABI 6100, for rapid extraction of DNA and RNA from bovine cells, bacterial broth cultures, and colonies lifted from agar

plates. While the procedure was second only to the Xtra Amp tubes in terms of speed and ease of use, the RNA yields were disappointingly small compared to those achieved with the Qiagen Viral RNA kit, and we were unable to detect BEV RNA in the 6100-derived samples. Results from extractions of bacterial cultures and colonies were more satisfactory, but overall we feel that the expense of acquiring and operating the ABI 6100 is not justifiable save when a laboratory contemplates extracting large numbers of samples per day.

We will continue to investigate some of the methods and technologies used in this project, particularly CFC for recovery of oocysts from source water samples. We hope to assay recovered oocysts by techniques other than nested PCR and IFA microscopy; some candidate methods are fluorescence in situ hybridization (FISH) and single tube RT-PCR, perhaps using the hsp70 gene as target. We are also interested in evaluating new methods for extraction of RNA from samples that provide the quality and quantity of RNA yielded by the Qiagen Viral RNA kit, with the economy and ease of use of the Xtra Amp tubes.

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